Near-infrared Spectroscopic Method to Identify *Cotesia flavipes* and *Cotesia sesamiae* (Hymenoptera: Braconidae)

T. J. COLE,[1] M. S. RAM,[2] F. E. DOWELL,[2] C. O. OMWEGA,[3] W. A. OVERHOLT,[3, 4] AND S. B. RAMASWAMY

Department of Entomology, Kansas State University, Manhattan, KS 66506

Ann. Entomol. Soc. Am. 96(6): 865–869 (2003)

ABSTRACT Parasitoids of the *Cotesia flavipes* complex (*C. flavipes* and *Cotesia sesamiae*) are natural enemies of stemboring lepidopteran pests in sub-Saharan Africa. The two species are difficult to differentiate using morphological markers, and a quick, reliable test was sought for their correct identification. After numerous, unsuccessful attempts at developing species-specific monoclonal antibodies that could differentiate between the two species, we were successful in using near-infrared spectroscopy to distinguish the cocoons of the two species with an accuracy of better than 85%. Calibrations were established using partial least squares analysis, enabling identification of cocoons of known species, cocoons from an alternative host, as well as in blind tests. This technology would greatly expedite identification of field-caught insects used to determine ecological parameters and parasitization rates of an individual species.

KEY WORDS species identification, near-infrared spectroscopy, parasitoids, *Cotesia flavipes*, *Cotesia sesamiae*

LARVAE OF LEPIDOPTERAN STEMBORERS such as *Chilo partellus* Swinhoe, *Chilo orichalcociliellus* Strand (Lepidoptera: Crambidae), *Sesamiae calamistis* Hampson, and *Busseola fusca* Fuller (Lepidoptera: Noctuidae) are generally considered the most damaging insect pests to gramineous crops in sub-Saharan Africa (Mohyuddin and Greathead 1970, Mohyuddin 1971, Polaszek and Walker 1991, Kfir 1995). With the exception of *C. partellus*, which was introduced from Asia in the 1930s, all are indigenous to Africa. Biological control of these pest species using endoparasitic wasps of the *Cotesia flavipes* species complex (Hymenoptera: Braconidae) has been assessed (Mohyuddin 1990, Polaszek and Walker 1991, Overholt et al. 1994, Ngi-Song et al. 1998). The complex is comprised of three putative species, *C. flavipes* Cameron (indigenous to Indo-Australia), *Cotesia sesamiae* Cameron (indigenous to sub-Saharan Africa), and *Cotesia chilonis* Matsumura (indigenous to Japan and China), all of which have been imported and released in various control programs in >40 countries (Polaszek and Walker 1991). Introduction of a given species has also occurred where another was known to be endemic, most notably, the release of *C. flavipes* into several African countries in which *C. sesamiae* was recorded (Mohyuddin 1971, Brenière and Bordet 1982, Skoroszewski and Van Hamburg 1987, Overholt et al. 1997, Schultess et al. 1997). *Cotesia flavipes* is now well established in Kenya, where it is the most abundant parasitoid of *C. partellus* (Zhou and Overholt 2001, Zhou et al. 2001).

Unfortunately, determination of the efficacy of individual parasitoid species has been hampered by difficulty in identification because the species are closely related and morphologically similar (Kimani and Overholt 1995, Kimani-Njogu and Overholt 1997, Overholt et al. 1997). The availability of reliable, diagnostic morphological markers is debatable (Polaszek and Walker 1991). In addition, although electrophoretic analysis of isozymes can differentiate the three species (Kimani-Njogu et al. 1998), a more rapid, yet low-cost and technologically simple test was sought. Smith and Kambhampati (1999) used sequences from mitochondrial 16S rRNA and NADH1 dehydrogenase genes to infer phylogenetic differences, but, ideally, our objective was to validate a non-molecular protocol to (1) reduce costs and (2) allow for the large sample sizes encountered in field

This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

[1] Current Address: Centre for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom.

[2] USDA–ARS, Grain Marketing and Production Research Center, Manhattan, KS 66502.

[3] International Centre for Insect Physiology and Ecology, Nairobi, Kenya.

[4] Current address: Indian River Research and Extension Center, University of Florida, Fort Pierce, FL 34945.

0013-8746/03/0865–0869$04.00/0

population sampling. Considering these requirements, we initiated experiments to develop a monoclonal antibody-based diagnostic method to different *C. flavipes* and *C. sesamiae.* In preliminary studies, we were unable to obtain monoclonal antibodies with the specificity needed to differentiate the two species (Cole and Ramaswamy, unpublished data). Herein, we describe the possibility of using near-infrared spectroscopy (NIRS) as a means of distinguishing *C. flavipes* and the *C. sesamiae-C. chilonis* subcomplex. *Cotesia chilonis* alone was not examined, because we were addressing a problem of sub-Saharan Africa, where *chilonis* does not occur. NIRS has been used successfully to identify stored-grain insect species (Dowell et al. 1999), to detect insects hidden in individual wheat kernels (Dowell et al. 1998), and to detect parasitized weevils (Baker et al. 1999) and parasitized fly puparia (Dowell et al. 2000).

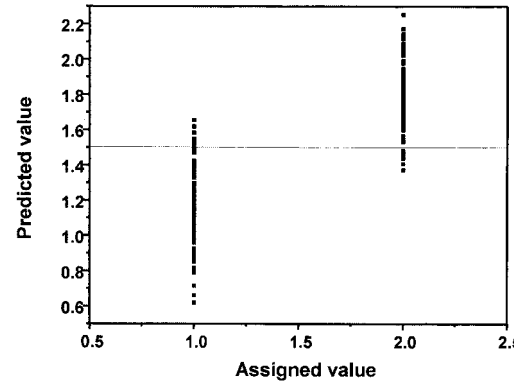

Fig. 1. Actual versus predicted value, using five factors in PLS cross-validation of NIRS data 800-1690 nm. A total of 200 cocoons were scanned, 25 insects of each species were scanned alternately. Each square represents one sample and the threshold value for classification, y = 1.5, separates the two species; values of >1.5 denote *C. sesamiae* and those of <1.5 denote *C. flavipes.* For both species, correct classification was 91%.

Materials and Methods

Insects. Insects were taken from cultures maintained at the International Centre for Insect Physiology and Ecology, Nairobi, Kenya. *Cotesia flavipes* was imported from Pakistan in 1992. *Cotesia sesamiae* was obtained from field-collected material reared from *B. fusca,* near Kitale in Western Kenya. Integrity of the parasitoid cultures was monitored regularly by examination of genitalia according to Kimani-Njogu and Overholt (1997). The host species *C. partellus* was reared as described previously by Overholt et al. (1994) and parasitized by adult wasps of *C. flavipes* or *C. sesamiae.* Stemborers were maintained on artificial diet at ≈25°C, 65–70% humidity. All insect specimens were frozen and shipped from Kenya via courier express to Kansas. Parasitoid larvae were dissected from hosts of various ages postparasitism; cocoons were gently teased from the cocoon mass using forceps. Note that for all investigations, the cocoons contained pupae. One hundred *C. flavipes* and 100 *C. sesamiae* cocoons, and 100 adult wasps were scanned with the near-infrared spectrometer (see below). Pilot studies had revealed that the most reproducible near-infrared spectra were obtained after insects had been dried for 2 h in a vacuum-sealed desiccator containing silica gel. All insects were scanned individually.

A set of 69 *C. flavipes* and 79 *C. sesamiae* cocoons, reared from *Diatraea saccharalis* F. (Crambidae) and supplied by R. Wiedenmann and M. Alleyne (Illinois Natural History Survey, Champaign, IL), were subjected to NIRS in blind trials.

Near-Infrared Spectroscopy. Specimens were placed longitudinally in a V-shaped black plastic trough (12 mm × 10 mm × 5 mm depth), which was illuminated with white light from a fiber bundle positioned 13 mm from the top of the trough and oriented at a 45° angle. A diode-array spectrometer (DA7000, Perten Instruments, Springfield, IL) was used to measure visible (400–750 nm) and near-infrared (750–1700 nm) reflectance. All spectra were relative to a background spectrum taken using the empty trough, which was found to have uniform absorption in the near-infrared range and negligible specular reflection. Collection of 15 spectra per insect required ≈3 s, thus an average spectrum was calculated for each insect. The time for acquisition of spectral data for 200 samples was <2 h. A baseline was collected with the black sample bucket empty, at the start of sample data collection.

Data Analysis. Data were analyzed using partial least squares (PLS) regression (Martens and Naes 1989) and GRAMS software (Galactic Industries, Salem, NH). Correlation plots were obtained to determine intraspecific and interspecific variation. Two-way comparisons of *C. flavipes* and *C. sesamiae,* arbitrarily assigned a value of 1 = *flavipes,* 2 = *sesamiae,* were used to construct calibration curves. Blind tests using insects of unknown species were subjected to a specific rejection threshold value of 1.5.

The accuracy of species identification was determined using coefficients of determination (r^2), standard errors of cross validation, and percentage of insects correctly classified. The regions sensitive to differences between the two species were estimated using the beta-coefficients and difference spectra. The value of the beta-coefficient indicates the importance of the wavelength for differentiation. Difference spectra were also plotted, which gives additional information on possible regions of interest.

Results and Discussion

Initial analyses indicated that it was easier to determine species when using parasitoid cocoons than when using parasitized hosts, parasitoid larvae, or adult wasps. Preliminary data from parasitized hosts showed evidence of being able to distinguish parasit-

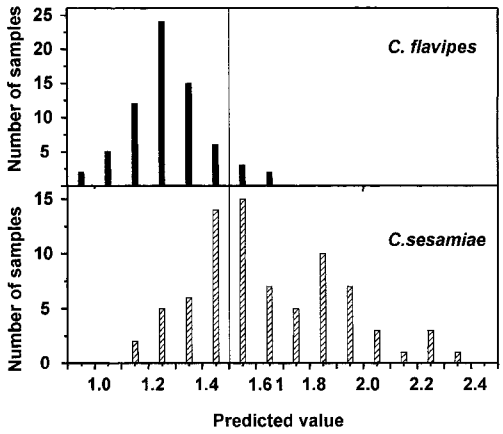

Fig. 2. Frequency distribution showing results of the blind trial using *C. flavipes* and *C. sesamiae* cocoons *ex. D. saccharalis.* Predicted values were calculated using the PLS cross-validation model in Fig. 1 (Note: the data in Fig. 1 were generated using cocoons *ex. C. partellus.*). For *C. flavipes*, 69 cocoons were scanned, yielding a predicted mean value of 1.27 (standard deviation = 0.14) and 85% correct classification. For *C. sesamiae*, 79 cocoons gave a predicted mean value of 1.65 (standard deviation = 0.28), 84% correct classification. The vertical line at predicted value 1.5 indicates the threshold value for classification.

ized versus nonparasitized larvae, although the parasitoid species could not be resolved. A distinct advantage of using near-infrared is that dead insects can be sampled, and fresh material is not necessary. Insects may be shipped to a laboratory for analysis, without concern for degradation, unlike, for example, when starch gel electrophoresis is employed. The ability to identify cocoons from the field further extends the utility of the method: in terms of field collection, cocoons are often more easily sampled, especially late in the season, than adult parasitoids. Moreover, spectra from cocoons may have proved most reliable because of variable water content in other stages, because the cocoon material is relatively dry. Cocoons were also easier to obtain (no dissection required) and easier to handle than individual parasitoid larvae.

Cross-validation data suggested that the average rates of classification for *C. flavipes* versus *C. sesamiae* cocoons were 91% each (Fig. 1). The results suggest that the determination of species need not be carried out on live insects and that insects may be transported as cocoons for several days without detrimental effects (e.g., Nairobi, Kenya to Manhattan, KS, was usually 5–7 d). Furthermore, we were able to differentiate three sets of cocoons with ≈84% accuracy in a blind test (Fig. 2); these parasitoids were reared in a host native to North America, *D. saccharalis*, rather than *C. partellus*, indicating that the calibration curve obtained using cocoons that egressed from *C. partellus* may be valid to differentiate parasitoids whose provenance may not be known, and in determining parasitoids harbored by different host lepidopterans from the same geographical location.

Inclusion of wavelengths in the visible region did not reduce the number of factors needed for classification and did not enhance the information gained in the near-infrared region. Using difference spectra (data not shown), PLS correlation plots, and beta-coefficients (Fig. 3), wavelengths of ≈945, 960 nm (water, third overtone); 1120, 1170 nm (CH_2, third overtone); 1275, 1390 nm (CH_2, second overtone); 1450 nm (water, first overtone; amides, first overtone; CH, first overtone; several combination bands occur between 1400 and 1600 nm); 1540, 1690 nm (CH_3 first overtone) were important in species determination. Such absorbances may correspond to differences in

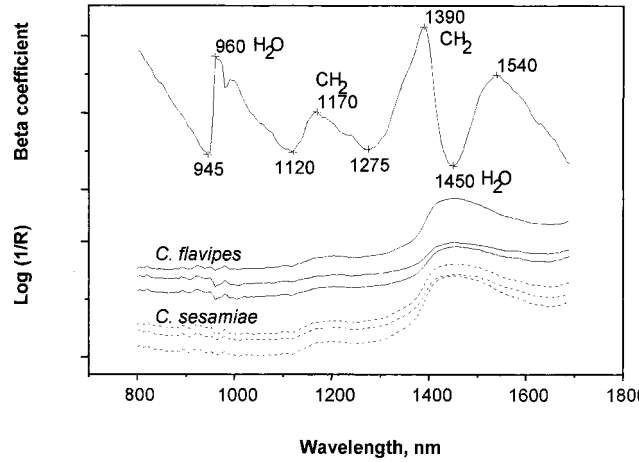

Fig. 3. Upper plot: Beta-coefficients versus wavelength for the PLS analysis of NIRS data of *C. flavipes* and *C. sesamiae* cocoons. Lower plot: near-infrared spectra of absorbance (log[1/R]) versus wavelength for *C. flavipes* (solid lines) and *C. sesamiae* (dashed lines). Each spectrum is the average of 10 samples, three sets for each species are shown.

moisture, lipids, or chitin between the two species. The water and CH_2 overtones, however, are more suggestive of possible differences in cuticular hydrocarbons or proteins, rather than chitin. Cuticular hydrocarbons have been implicated in insect species classifications (Dowell et al. 1999). Further work is required to identify the biochemical nature of the distinguishing molecules and, thus, to gain a better insight into the specific wavelengths used in classifications. In preliminary studies using infrared (IR) and Raman spectroscopy, some differences were observed that support the near-infrared findings. Vibrations as a result of lipid CH_2 groups were prominent in the IR spectra; vibrations as a result of amides were prominent in Raman spectra.

Diode-array technology is now comparatively inexpensive, and portable instruments are available for field studies. At present, differentiation of field specimens involves collection of potential hosts (i.e., both parasitized and nonparasitized) and rearing of the parasitoids to the adult stage, before being subjected to identification by expert staff. In contrast, the near-infrared analysis used cocoons, saving time in waiting for adult emergence; moreover, once calibrated for *C. flavipes* and *C. sesamiae*, non-specialists and non-spectrometrists could differentiate insect species using a near-infrared spectrometer. Initial costs of purchase of the spectrometer (<$10,000; portable machines approximately $5,000) are far less expensive than setting up an analytical molecular laboratory, and running costs would be substantially reduced, compared with the consumable overheads for DNA/RNA analysis. The expenditure of running an near-infrared analysis, in comparison with morphologic studies, would be outweighed by (1) higher through-put of data, (2) ease of identification, and (3) because parasitized and nonparasitized hosts could be separated, the time and cost of maintaining nonparasitized insects would be avoided. Finally, near-infrared spectrometry instrumentation currently available on the market is rugged and is able to withstand field use. This latter feature is particularly important in the context of sub-Saharan Africa where *C. flavipes* and *C. sesamiae* are used as biocontrol agents.

Acknowledgments

We thank Jim Throne (USDA–ARS GMPRC, Manhattan, KS), Jim Nechols (Department of Entomology, Kansas State University, Manhattan, KS), and Rob Wiedenmann (Illinois Natural History Survey, Champaign, IL) for comments. Funded in part by United States Agency for International Development Grant Number PCE-G-00-98-00044-00 under the University/International Agricultural Research Centers Linkages Program (G/EGAD/AFS-98-001). This is paper number J03-98-J, Kansas State University Agricultural Experiment Station. Voucher specimens have been placed in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Lot Number 139.

References Cited

Baker, J. E., F. E. Dowell, and J. E. Throne. 1999. Detection of parasitized rice weevils in wheat kernels with near-infrared spectroscopy. Biol. Control 16: 88–90.

Brenière, J., and D. Bordet. 1982. Interêt et difficultés de la lutte biologique par introduction d'entomophage – cas des foreurs du riz en Afrique. Agronomie Tropical 37: 203–206.

Dowell, F. E., A. B. Broce, F. Xie, J. E. Throne, and J. E. Baker. 2000. Detection of parasitized fly puparia using near infrared spectroscopy. J. Near Infrared Spectr. 8: 259–265.

Dowell, F. E., J. E. Throne, and J. E. Baker. 1998. Automated nondestructive detection of internal insect infestation of wheat kernels by using near-infrared reflectance spectroscopy. J. Econ. Entomol. 91: 899–904.

Dowell, F. E., J. E. Throne, D. Wang, and J. E. Baker. 1999. Identifying stored-grain insects using near-infrared spectroscopy. J. Econ. Entomol. 92: 165–169.

Kfir, R. 1995. Parasitoids of the African stemborer, *Busseola fusca* (Lepidoptera: Noctuidae) in Natal, South Africa. African Entomol. 2: 67–68.

Kimani, S. W., and W. A. Overholt. 1995. Biosystematics of the *Cotesia flavipes* complex (Hymenoptera: Braconidae): interspecific hybridization, sex pheromone and mating behaviour studies. Bull. Entomol. Res. 85: 379–386.

Kimani-Njogu, S. W., and W. A. Overholt. 1997. Biosystematics of the *Cotesia flavipes* species complex (Hymenoptera: Braconidae), parasitoids of the gramineous stemborers. Insect Sci. Applic. 17: 119–130.

Kimani-Njogu, S. W., W. A. Overholt, J. B. Woolley, and C. O. Omwega. 1998. Electrophoretic and phylogenetic analyses of selected allopatric populations of the *Cotesia flavipes* complex (Hymenoptera: Braconidae), parasitoids of cereal stemborers. Biochem. Syst. Ecol. 26: 285–296.

Martens, H., and T. Naes 1989. Multivariate calibrations. Wiley, Chichester, United Kingdom.

Mohyuddin, A. I. 1971. Comparative biology and ecology of *Apanteles flavipes* and *A. sesamiae* Cam., as parasites of gramineous borers. Bull. Entomol. Res. 61: 33–39.

Mohyuddin, A. I. 1990. Biological control of *Chilo* spp. in maize, sorghum and millet. Insect Sci. Applic. 11: 721–732.

Mohyuddin, A. I., and D. J. Greathead. 1970. An annotated list of parasites of gramineous stemborers in East Africa, with a discussion on their potential in biological control. Entomophaga 15: 241–274.

Ngi-Song, A. J., W. A. Overholt, and R. Stouthamer. 1998. Suitability of *Busseola fusca* and *Sesamiae calamistis* (Lepidoptera Noctuidae) for the development of two populations of *Cotesia sesamiae* (Hymenoptera: Braconidae) in Kenya. Biol. Control 12: 208–214.

Overholt, W. A., A. J. Ngi-Song, C. O. Omwega, S. W. Kimani-Njogu, J. Mbapila, M. N. Sallam, and V. Ofomata. 1997. A review of the introduction and establishment of *Cotesia flavipes* Cameron in East Africa for biological control of cereal stemborers. Insect Sci. Applic. 17: 79–88.

Overholt, W. A., K. Ogeda, and P. Lammers. 1994. Distribution and sampling of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) in maize and sorghum in Kenya. Bull. Entomol. Res. 84: 367–378.

Polaszek, A., and A. K. Walker. 1991. The *Cotesia flavipes* species complex; parasitoids of cereals and stemborers in the tropics. Redia 74: 335–341.

Schultess, F., N. A. Bosque-Pérez, A. Chbi-Olaye, S. Gounou, R. Ndemah, and G. Goergen. 1997. Exchange of natural enemies of lepidoteran cereal stemborers between African regions. Insect Sci. Applic. 17: 97–108.

Skoroszewski, R. W., and H. Van Hamburg. 1987. The release of *Apanteles flavipes* (Cameron) (Hym: Braconidae) against stemborers of maize and grain-sorghum in South Africa. J. Entomol. Soc. Africa 50: 249–255.

Smith, P. T., and S. Kambhampati 1999. Status of the *Cotesia flavipes* complex (Braconidae: Microgastrinae) based on mitochondrial rRNA and NADH1 dehydrogenase gene sequences. J. Kansas Entomol. Soc. 72: 306–314.

Zhou, G., J. Baumgartner, and W. A. Overholt. 2001. Impact of an exotic parasitoid on stemborer (Lepidoptera) population dynamics in Kenya. Ecol. Appl. 11: 1554–1562.

Zhou, G., and W. A. Overholt. 2001. Spatial-temporal population dynamics of *Cotesia flavipes* (Hymenoptera: Braconidae) in Kenya. Environ. Entomol. 30: 869–876.

Received for publication 16 September 2002; accepted 3 June 2003.